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A method of analyzing the intestinal flora by means of antibodies labeled with fluorescence (labeling with 1-dimethylamino-naphtalene-sulfonic acid-5).

by F. Petuely and G. Lindner.

Translated from Schweiz. Z. Path. Bakt. 22: 747-751 (1959) by the Technical Library, Technical Information Division.

Critical reflection shows our knowledge of human intestinal flora to be surprisingly inadequate. Although it was demonstrated recently that the percentage of *Bacterium coli* present in the feces is small (contrary to earlier assumptions), its actual composition is still unknown. Haenel, in particular, showed that relatively large amounts of anaerobic, grampositive rods can be grown even from the stools of adults, which initially were named *Lactobacillus bifidus* pending further classification. For this reason the author speaks of a bifidus flora of adults and believes that the feces of infants differs from those of adults only by the absence of proteolytes. Besides the studies of Haenel, those of Herrmann deserve mention. He succeeded in culturing a large number of gramnegative, anaerobic rods from stools, consolidated under the collective term "bacterioides group." However, neither Haenel nor Herrmann, or any other investigators of intestinal bacteria are able to grow more than 1 to 2% of the organisms present in the stool, which means that no-one knows whether the remaining 98 to 99% are dead or cannot be cultivated with currently available methods.

The bacteriologist therefore lacks a suitable method for the visualization of the actual number of various bacteria in the stool, whether dead or alive, and cannot gain insight into the true relations between these organisms; in short, he cannot carry out a total analysis.

We pointed out during last year's symposium on the problems of intestinal bacteriology held at Graz that, in our opinion, the method of differential staining with fluorescent antibodies represents the only possibility for obtaining genuine data on the composition of intestinal flora.

We have, in collaboration with Lindner, developed a quantitative method for the analysis of bacterial mixtures, especially those of the stool, and have already applied it to the analysis of infant feces.

The principle of our method involves cultivation of the various bacterial species or strains from the stools, production of antisera and their linkage to a fluorescent dye. The labeled antisera are used in differential staining of stool smears. The percentage of specifically stained bacteria on the smear is determined by observation or photography under ultraviolet light, followed by visualization of the same field of view in visible light under dark field illumination. The absolute content of specifically stained organisms in the stool and their growth rate during culture may be established by determining the absolute bacterial count in a chamber and by quantitative studies of cultures. Thus a method is finally given which offers information on the value of germ counts in selective media.

I should like to describe the specific method of our process by way of analysis of an infant's stool. A precisely weighed amount of feces is suspended 1:100 in distilled water as homogeneously as possible with the aid of a high-revolution agitator, from which additional dilutions up to 1:100 million are prepared. The absolute germ count is obtained from the 1:1000 dilution in Helber's counting chamber. The higher dilutions are used in quantitative growth of the different bacterial species and, especially, of *Lactobacillus bifidus*. Smears are prepared from the primary dilution 1:100 for gram staining and for differential staining with fluorescent antibodies. These shall be discussed in detail below. For the purpose of obtaining antisera, the various organisms grown on selective media are subjected to pure culture. Labeling of antisera is accomplished with 1-dimethylamino-naphtalene-sulfonic acid-5 introduced by Mayersbach. This acid has been particularly acceptable due to the simple handling involved and its low tendency to non-specific staining. Acting on our recommendation, the firm of Fluka, Buchs, has released it commercially as an acid chloride which is suitable for direct linkage.

Stool smears slated for differential staining must be subjected to special treatment prior to incubation with the serum. We were able to show that staining (e.g., gram staining) tends to wash off organisms from stool smears, particularly gramnegative germs. This produces an excess of grampositive organisms and distorts bacterial relations. In order to prevent this shift in relations, we cover the smears chosen for gram staining with a celloidin layer, and those prepared for fluorescence analysis with a film of hardened gelatine. Staining proceeds through the protective layers and the quality of visualization is not impaired.

The quantitative evaluation of stool smears was initially beset with extraordinary difficulties. These consisted first in the visualization of bacteria in the dark field with visible light and, secondly, in the photography of the same field of view under UV and visible light. The difficulty inherent in dark field representation in visible light is due to the amorphous detritus of stool substance which has an index of refraction similar to that of bacteria. Inclusion agents must be used

that penetrate the detritus and thus allow differentiation of bacteria. Water, which would be particularly suitable, causes extinction of fluorescence. Testing of about fifty different inclusion agents has led to selection of certain ones possessing special powers of penetration without disturbing fluorescence. Methanol, acetone and acetic anhydride have proved to be especially feasible.

Quantitative evaluation is carried out by mounting both photographic negatives of a field together in a dual frame and projecting them on the wall. Direct counting of fluorescent organisms under the microscope is impossible since they are decolorized during the long period of time required for this task.

The results of quantitative evaluation of two infant stools are listed in the table below. Only one antiserum was used for the specific infant bifidus. It is evident that the infant indeed possesses a nearly pure bifidus flora represented by a single strain, contrary to recent assertions.

We hope to apply this method also to procurement of information about the true composition of adult intestinal flora. We already found that no infant bifidus strains are demonstrable in the stool of nursing mothers.

Table

	<u>Child #1</u>	<u>Child #2</u>
Age	6 days	7 days
pH of the stool	4.8	4.4
Absolute germ count per gram	$292.6 \pm 25.7 \times 10^9$	$126.6 \pm 17.8 \times 10^9$
Gram-stained smear, positive rods w/bifidus morphology	$99.2 \pm 0.7\%$	$98.5 \pm 0.9\%$
<u>Specific infant bifidus</u>		
Fluorochromed smear	$96.4 \pm 0.8\%$	$97.0 \pm 1.0\%$
Culture	24,000 millions	12,000 millions
Rate of growth	8.5%	10.0%

Illustration

Infant. 6 days old. 95.4% grampositive rods in the gram-stained stool smear. Bifidus flora fully developed. Upper frame: Fluorescent dark field exposure upon staining with fluorescence-labeled anti-bifidus serum. Lower frame: Same field in dark field illumination with visible light. Nearly all organisms give off fluorescence.

Conclusion

It is true that the diversity of intestinal bacteria is enormous. I believe, however, that our method permits identification of the dominant organisms. It was assumed until recently that *Bacterium coli* has the paramount role in the intestinal flora. Lately some authors have asserted that *Lactobacillus bifidus* predominates. A decision on the actual ratio of organisms in adult stools may be reached only upon total analysis of intestinal flora. We believe that our method is suited for this purpose. The culture of dominant organisms and the production of antisera does not seem to present difficulties. For the time being, bacteria whose share in the feces is less than 1% are not important. When individuals of the same housing community (i.e., from the same milieu) are examined, the sera of one person may probably serve for stool analyses of others, so that the effort involved is small and compares with that expended in connection with the two infants examined by us.